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Protein phosphatase 4 negatively regulates LPS cascade by inhibiting ubiquitination of TRAF6☆

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Abstract TRAF6 is an E3 ubiquitin ligase that transduces signals from members of the TLR/IL-1R family. Multiple molecules have been found to associate with TRAF6 and exert their functions in this pathway. Herein, by yeast two-hybrid screen using TRAF6 as bait, we identified PP4 as a potential TRAF6-interacting protein. PP4 physically interacted with TRAF6 and was recruited to TLR4 complex upon LPS stimulation. PP4 negatively regulated LPS-induced and TRAF6-mediated NF-κB activation by inhibiting the ubiquitination of TRAF6. LPS stimulation also induced the expression of PP4. Taken together, our findings suggest that PP4 is a negative feedback regulator of LPS/TLR4 pathway.

Keywords: Protein phosphatase 4; Tumor necrosis factor receptor-associated factor 6; Lipopolysaccharide; Nuclear factor-κB; Ubiquitination

1. Introduction

The innate immune response in vertebrates is the first line of defense against infectious pathogens. In innate immune system, different phagocytes such as neutrophils, macrophages, and dendritic cells play crucial roles in discriminating between pathogens and self by utilizing signals from the toll-like receptors (TLRs) [1,2]. LPS serves as a ligand specific for TLR4. Ligation of LPS to TLR4 leads to signal activation via downstream signaling factors, which includes the adaptors MyD88, IRAKs, TRAF6 and TAK1, and thereafter induce the nuclear transport of NF-κB and the activation of a set of MAPKs [2,3]. TRAF6 is essential for the signal activation, as TRAF6 deficiency results in defective LPS signaling [4]. Similar to most TRAF proteins, TRAF6 is composed of a highly conserved TRAF-C domain, a TRAF-N domain and a more variable amino-terminal domain that contains a RING-finger and several Zn fingers [5]. TRAF6 has been demonstrated to be a RING-finger-dependent E3 ubiquitin ligase which is itself activated by ubiquitination through a K63-ubiquitinated mechanism [6,7].

The activation of TLRs can effectively protect the host, but sustained activation may lead to the development of chronic inflammatory disorders. To prevent detrimental and overactive inflammatory responses, TLR signaling must be tightly controlled. So far, the uncovered negative regulation underlying the TLRs signaling can be divided into five levels [8]. Recent studies by us and others have identified many intracellular inhibitors that negative regulates TLRs pathways, such as TOLLIP, MyD88s, IRAK-M, SOCS1, SOCS3, CYLD and A20 [8–10]. Among these regulators, SOCS1, A20 and CYLD have been demonstrated to inhibit the activation of TRAF6.

Protein phosphatase 4 is a protein serine/threonine phosphatase belonging to the PP2A phosphatase family. PP4 is highly conserved during evolution, suggesting that PP4, like PP1 and PP2A (the most conserved enzymes known in eukaryote evolution), might serve critical functions in vivo [11]. Previous studies have shown that PP4 involves in multiple cellular processes, including regulation of microtubule growth/organization, apoptosis, TNF-α signaling, thymocyte development, pre-T-cell receptor signaling, and the activation of JNK and NF-κB [12–16]. Herein, by yeast two-hybrid screening, we found PP4 physically associated with an important signal transducer TRAF6, and acted as a negative regulator in TRAF6- and LPS-mediated NF-κB activation through its inhibition on the ubiquitination of TRAF6. Together, all these data have defined a novel role of PP4 in the innate immune system.

2. Materials and methods

2.1. Antibodies and reagents

Anti-TLR4, anti-TRAF6, anti-FLAG, anti-HA, horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies and LPS were described previously [17]. Anti-Ub was purchased from Santa Cruz Biotechnology. Anti-PPP4C was purchased from ptglab. Anti-β-actin was from Abcam.

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Abbreviations: TRAF6, tumor necrosis factor receptor-associated factor 6; TLR, toll-like receptor; IL, interleukin; NF-κB, nuclear factor-κB; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; MyD88, myeloid differentiation primary response gene 88; IRAK, IL-1 receptor-associated kinase; TAK1, transforming growth factor-β-activated kinase-1; TOLLIP, toll-interacting protein; SOCS, suppressor of cytokine signaling; IKK, IκB kinase; TNF, tumor necrosis factor; TAB, TAK1 binding protein; CSF, colony-stimulating factor; CYLD, cylindromatosis; TCR, T-cell receptor

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2.2. cDNA and plasmids

PP4 cDNA was amplified by PCR, then cloned into pCMV-Tag2 (Stratagene), pGBKT7 and pGADT7 (Clontech) vectors, respectively. Murine TRAF6 was cloned into pGBKT7, pGADT7 and pCMV-HA (Clontech). The TRAF6 mutants were amplified by PCR.

2.3. Yeast two-hybrid system

Yeast two-hybrid screens were performed using the Matchmaker GAL4 Two Hybrid System 3 (Clontech) and the human fetal brain Matchmaker cDNA library (Clontech) following the manufacturer's instructions. The same system was used for detecting the interaction of two proteins following the manufacturer's instructions.

2.4. Cell culturing, transient transfection, and treatment

The murine RAW264.7 cells were grown, stimulated and transfected as described before [17]. HEK293T cells were cultured in DMEM (Hyclone) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin. Transfections were carried out using Lipofectin 2000 (Invitrogen) according to the manufacturer's instructions.

2.5. NF-κB assay

Cells were transfected with 100 ng of the pNF-κB luciferase reporter (Stratagene) and 10 ng of the pRL-TK *Renilla* reporter (Promega), in combination with the indicated expression plasmids. Cells were harvested after the indicated periods of time, and the firefly luciferase levels were determined using the Dual-Luciferase Reporter Assay System (Promega). The level of *Renilla* luciferase activity was used to normalize the transfection efficiencies. The luciferase assay was done in triplicate, and the results are shown as means ± standard errors (S.E.).

2.6. RNA isolation and RT-PCR

Total cellular RNAs were extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The cDNA was reverse transcribed from 1.0 μg total RNA with oligo dT using the M-MLV Reverse Transcriptase (Promega) as recommended by the supplier. Subsequently, the cDNA preparations were used as the templates for PCR to amplify GAPDH and PP4.

2.7. Immunoblotting and immunoprecipitation

Cells were lysed with MPER Protein Extraction Reagent (Pierce), and equal amounts of the samples were loaded for SDS–PAGE. Immunoblotting was carried out on nitrocellulose membranes (Pharmarcia) and the samples were detected using an ECL reagent (Pierce). Immunoprecipitation was carried out using a MPER Immunoprecipitation Kit (Pierce).

2.8. RNA interference

The RNAi pSilencer system (Ambion) was used. The different pSilencer recombinant vectors were constructed by inserting 64-mer synthetic DNA oligonucleotides that encode two 19-nucleotide (nt) reverse complements with homology to a portion of the target gene. For the mPP4 siRNA construct, target sequence was nt 91–109. For the EGFP siRNA construct, the target sequence was nt 158–176.

3. Results

3.1. Interaction ofPP4 with TRAF6

In an attempt to identify novel proteins involved in TRAF6-related signaling pathway, we performed yeast two-hybrid screens with full length murine TRAF6 as bait and a human fetal brain cDNA library as target. By screening and β-galactosidase assays we obtained a cDNA encoding a 151-amino acid protein identical to the C-terminus (the 157–307 amino acids) of PP4. To further confirm whether the full-length PP4 could also interact with TRAF6, we constructed various GAL4 DNA-binding domain (BD) and GAL4 transcription activation domain (AD) fusion proteins as shown in Fig. 1A. Both BD-TRAF6/AD-PP4 and BD-PP4/AD-TRAF6 expressing AH109 transformants turned out to be positive in β-galactosidase assays, and we concluded that PP4 might be a potential TRAF6-binding protein.

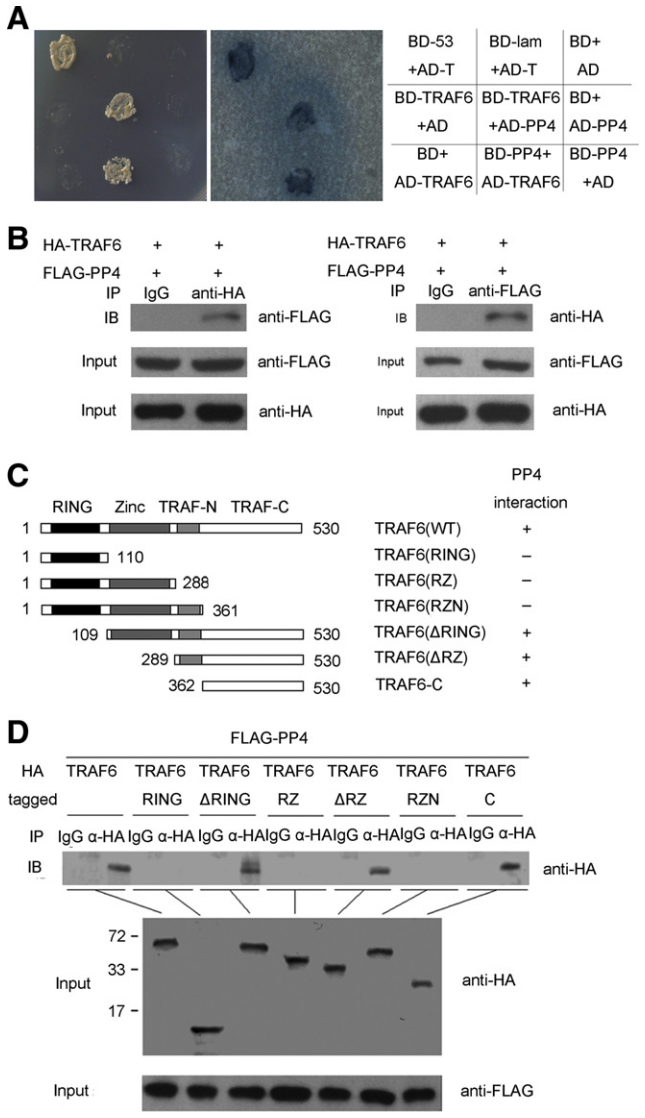

Fig. 1. Interaction of PP4 and TRAF6. (A) Interaction between PP4 and TRAF6 proteins in yeast. (B) TRAF6 interacts with PP4 in an over-expressed system in HEK293T cells. (C) A schematic presentation of TRAF6, its deletion mutants, and the interaction with wild-type PP4. (D) The TRAF-C domain of TRAF6 is required for its interaction with PP4.

In an effort to verify their interactions in vivo, we performed immunoprecipitation under over-expression conditions in HEK293T cells and found that FLAG-PP4 could co-precipitate with HA-TRAF6 (Fig. 1B). The results indicated that PP4 interacted with TRAF6 when over-expressed in cells.

TRAF6 is a multifunction protein with several domains. The RING-finger and zinc finger domains can mediate downstream signaling events and the TRAF-C domain is for protein–protein interaction [5]. To determine which domain of TRAF6 interacts with PP4, we constructed some truncated mutant forms of TRAF6 (Fig. 1C), and detected whether these mutant

proteins could also associate with PP4 in co-immunoprecipitation assays. Results shown in Fig. 1D suggested that the TRAF-C domain of TRAF6 was sufficient for its interaction with PP4.

3.2. PP4 is recruited to TLR4 complex upon LPS stimulation in RAW264.7 cells

As TRAF6 has been proved to be a pivotal signal transducer in LPS/TLR4 pathway, it is quickly recruited to the TLR4 complex upon LPS stimulation, then leading to the activation of NF-κB. To determine whether the endogenous PP4 is associated with TRAF6 and recruited into TLR4 complex, we performed endogenous co-immunoprecipitation experiments in RAW264.7 cells stimulated with or without LPS. As shown in Fig. 2A, PP4 was not associated with TRAF6 in unstimulated RAW264.7 cells, but their interaction emerged when the cells were treated with LPS. LPS treatment also induced the recruitment of PP4 into TLR4 receptor complex (Fig. 2B), suggesting that endogenous PP4 was recruited into TLR4 receptor complex in a LPS-stimulated manner.

3.3. PP4 inhibits LPS-induced and TRAF6-mediated NF-κB activation

The above data that PP4 could bind to TRAF6 in response to LPS ligation suggests that PP4 may be involved in LPS/TLR4 signaling cascade. To determine whether PP4 has regulatory function in LPS- and TRAF6-mediated NF-κB activation, we performed NF-κB luciferase reporter assays. As shown in Fig. 3A and B, these data indicated that PP4 suppressed LPS- and TRAF6-mediated NF-κB activation in a dose-dependent manner.

To further address the functional role of PP4 in the TLR4 signaling pathway, we used siRNA to knockdown the endogenous expression of PP4 in RAW264.7 cells. Western blot analysis indicated that the RNAi plasmid could inhibit the expression of endogenous PP4 in RAW264.7 cells, while the control RNAi plasmid had no effect on PP4 expression (Fig. 3C, left panel). The expression of FLAG-PP4 could also be knocked-down by pSilencer-PP4 (Fig. 3C, right panel). In reporter gene assays, PP4 knockdown by pSilencer-PP4 enhanced NF-κB activation by LPS or TRAF6 (Fig. 3D and E). All these data suggested that PP4 was a negative regulator for LPS/TLR4 signaling.

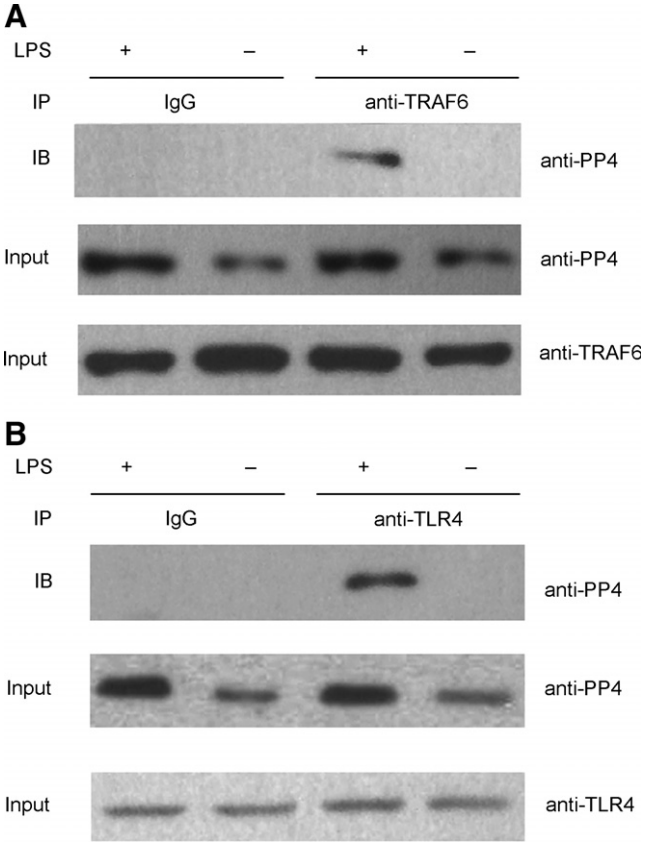

Fig. 2. PP4 is recruited to the TLR4 signaling complex after LPS stimulation. Wild-type RAW264.7 cells were unstimulated (−) or stimulated (+) with LPS (500 ng/ml). Cell lysates were immunoprecipitates (IP) with anti-TRAF6 (A), anti-TLR4 (B) or control antibody and immunoblotted with anti-PP4 antibody. Cell lysates were also probed with anti-TRAF6 (A), anti-TLR4 (B) or anti-PP4 antibody to analyze the protein expression.

3.4. PP4 exerts the negative role through suppressing the ubiquitination of TRAF6

It has recently been demonstrated that TRAF6 is an E3 ubiquitin ligase that is ubiquitinated by itself in response to LPS stimulation, and recycled via deubiquitination [11,18]. This polyubiquitination does not target proteins for degradation, but has been found to regulate DNA repair and protein kinase activation through a degradation-independent mechanism [19]. From the results above, we wondered the mechanism underlying the negative regulation of PP4 and whether PP4 can affect the auto-ubiquitination of TRAF6. We transfected RAW264.7 cells with expression plasmids for TRAF6, PP4 and PP4-RNAi as indicated in Fig. 4A. The results indicated that PP4 inhibits the ubiquitination of TRAF6.

To evaluate the effect of PP4 on endogenous TRAF6, we detected the ubiquitination of TRAF6 in wild-type RAW264.7 cells, PP4-introduced RAW264.7 cells and PP4 knocked-down RAW264.7 cells in response to LPS stimulation at indicated time. The results showed that in existence of PP4, the ubiquitinating levels of TRAF6 had been obviously reduced while with the absence of PP4, the ubiquitination of TRAF6 had been strengthened (Fig. 4B). These results did provide supportive envidence for our model that PP4 inhibited TRAF6-dependent activation of NF-κB likely by inhibiting the ubiquitination of TRAF6.

3.5. PP4 exerts negative regulation in a feedback loop in responding to LPS stimulation

Many genes involved in inflammatory response undergo changes in expression pattern after various stimuli. PP2A, another important phosphatase in PP2A family, can be induced by CSF in macrophages [20]. So we hypothesized that activation of TLR4 signaling by LPS induces PP4, which in turns leads to the inhibition of NF-κB. We stimulated wild-type RAW264.7 cells with LPS in different times and found that PP4 expression was apparently induced after 60 min stimulation and decreased to normal at 300 min after treatment (Fig. 5A). Consistent with the up-regulation in protein level, induction of PP4 by LPS was also observed by RT-PCR at the mRNA level (Fig. 5B). These results suggested that PP4 was a part of the negative feedback loop of TLR4 pathway.

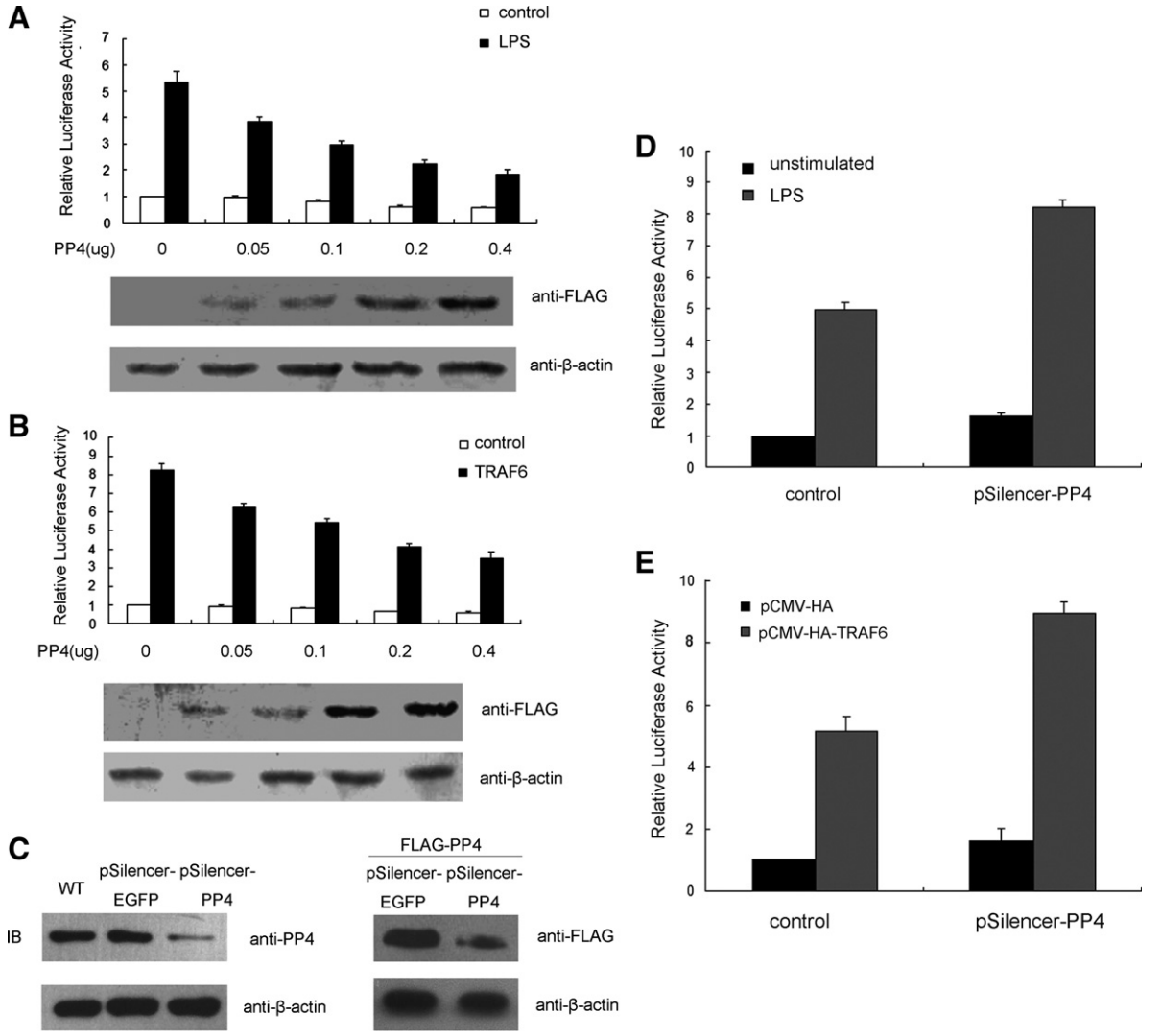

Fig. 3. Expression of PP4 reduces LPS- and TRAF6-dependent NF-κB activation. (A) PP4 inhibits LPS-induced NF-κB activation in a dose-dependent manner in RAW264.7 cells. RAW264.7 cells were transfected with expression plasmids for PP4 together with 100 ng of NF-κB luciferase and 10 ng of pRL-TK *Renilla* reporter plasmids. At 36 h post-transfection, the cells were treated with LPS (500 ng/ml) for 4 h, and then the cell lysates were collected and luciferase reporter assays were carried out. Cell lysates were also probed with anti-FLAG and anti-β-actin. (B) PP4 inhibits TRAF6-induced NF-κB activation in a dose-dependent manner in RAW264.7 cells. RAW264.7 cells were transfected with reporter plasmids and protein-encoding plasmids as indicated. Luciferase assays were carried out 36 h after transfection. Cell lysates were also probed with anti-FLAG and anti-β-actin. (C) PP4 knockdown by pSilencer-PP4 markedly reduced both endogenous (left) and exogenous (right) expression of PP4. (D) Wild-type and pSilencer-PP4 transfected RAW264.7 cells were stimulated with LPS (500 ng/ml) for 4 h or left untreated. Luciferase assays were carried out. (E) RAW264.7 cells were transfected with TRAF6 encoding or control plasmid together with or without pSilencer-PP4. Thirty-six hours after transfection, luciferase assays were carried out.

To examine whether the recruitment of endogenous PP4 to the TLR4 signaling complex is in a time-dependent manner, we stimulated RAW264.7 cells with LPS and used anti-PP4 antibody to precipitate the PP4 signaling complex. Endogenous TLR4 in the precipitated signaling complex was immunoblotted with anti-TLR4 antibody, and its amount peaked at 90 min after stimulation, and declined thereafter to the lowest point after 180 min. We also detected the TRAF6 in PP4 precipitation complex, their interactions appeared at 60 min after treatment and sustained until 180 min (Fig. 5C). Time kinetics revealed that the recruitment of PP4 to the receptor complex did not happen immediately after stimulation, but in a PP4-expression and LPS-stimulation dependent manner, suggesting that PP4 was a negative regulator in a feedback loop in LPS/TLR4 pathway.

4. Discussion

In this study, we have identified PP4 as a negative regulator of LPS/TLR4 signaling cascade that directly targets TRAF6 and suppresses its ubiquitination. Previous studies have revealed that TRAF6 is a critical modulator in LPS/IL-1, CD40, TCR and p75 pathways [4,5,21]. In an attempt to screen TRAF6 binding proteins, PP4 was identified as a

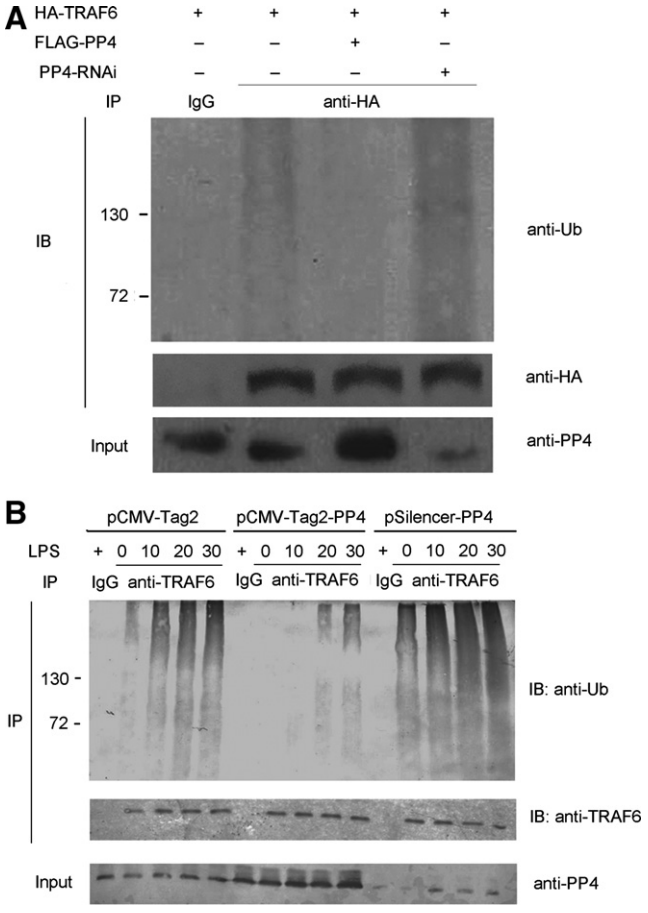

Fig. 4. PP4 inhibits the ubiquitination of TRAF6. (A) PP4 suppresses the ubiquitination of TRAF6. RAW264.7 cells were transfected with plasmids as indicated. Whole cell extracts were immunoprecipitated with anti-HA or control IgG, and immunoblotted with anti-Ub antibody. Whole cell lysates were also probed with anti-HA or anti-PP4 to analyze the expression of HA-TRAF6 or PP4. (B) Knockdown of PP4 enhances the ubiquitination level of TRAF6 in RAW264.7 cells. Wild-type, pCMV-Tag2-PP4 and pSilencer-PP4 transfected RAW264.7 cells were treated with LPS (500 ng/ml) for the indicated times. Cell lysates were immunoprecipitated with anti-TRAF6 or control IgG. Immunoprecipitates were immunoblotted with anti-Ub in order to detect ubiquitinated TRAF6 or anti-TRAF6 to detect TRAF6. Whole cell lysates were probed with anti-PP4 to analyze the protein level of PP4.

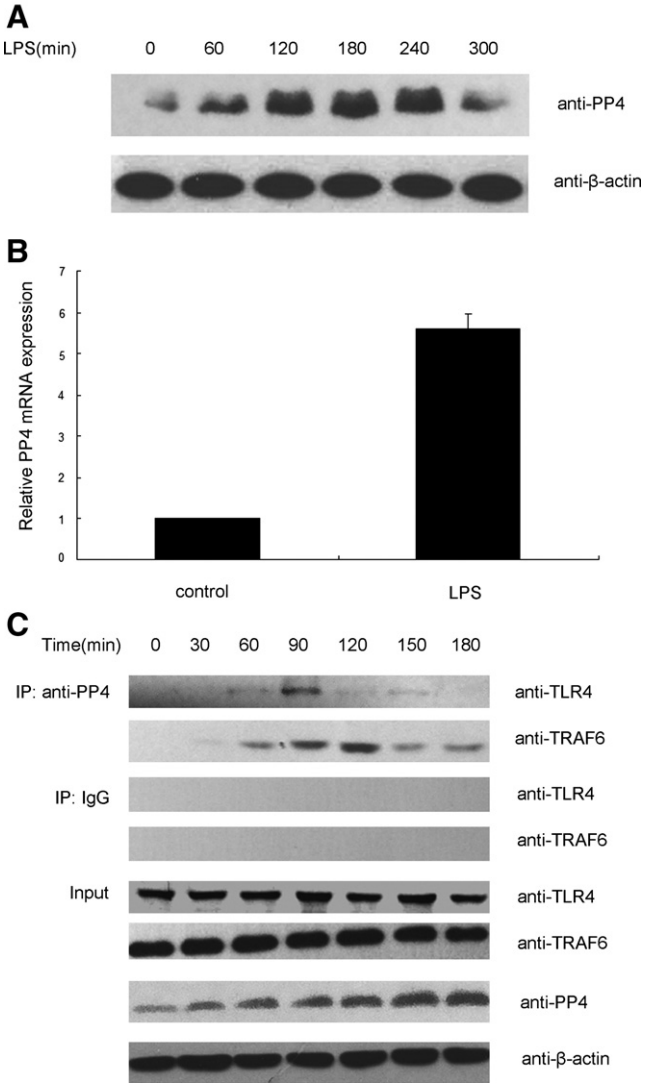

Fig. 5. PP4 exerts negative regulation in a feedback loop in responding to LPS stimulation. (A) LPS induces PP4 expression at the protein level in RAW264.7 cells. Wild-type RAW264.7 cells were treated with LPS (500 ng/ml) for the indicated times. Whole cell extracts were collected and probed with anti-PP4 antibody to analyze the protein level of PP4. (B) LPS induces PP4 expression at mRNA level in RAW264.7 cells. Wild-type RAW264.7 cells were treated with LPS (500 ng/ml) for 4 h. Total RNA was isolated and RT-PCR was carried out. The RT-PCR products of PP4 were detected by agarose gel electrophoresis. GAPDH was used as a control. Quantification of PP4 mRNA was measured by gel analysis software, normalized by calculating the ratio of the mRNA to GAPDH. (C) PP4 is recruited to TLR4 complex at 1 h after LPS treatment. RAW264.7 cells were stimulated with LPS (500 ng/ml) for the indicated times. Cell lysates were immunoprecipitated with anti-PP4 or control IgG antibody, and immunoblotted with anti-TLR4 or anti-TRAF6 antibody. Cell lysates were also probed with anti-TLR4, anti-TRAF6, anti-PP4 or anti-β-actin antibody.

TRAF6-interacting protein that interact with the TRAF-C domain of TRAF6. The suppressive role of PP4 in LPS-induced NF-κB activation and the direct association with TRAF6 lead us to suppose that PP4 may be an inhibitor regulating the activation of TRAF6 and LPS cascade. As TRAF6 is activated by K63-linked ubiquitination, it can also be inactivated by deubiquitination. By ubiquitnation assays, we found that PP4 acts as a negative regulator in ubiquitination of TRAF6 induced by LPS. Previous studies have identified A20 and CYLD, two deubiquinylating enzymes that accomplish their roles by directly removing ubiquitin moieties from TRAF6 [22,23]. We carried out in vitro deubiquitination assays, but failed to detect any deubiquinylating activity of PP4, revealing that PP4 acts through a distinctive mechanism. Our findings show that PP4 is also a LPS-inducible protein, because PP4 protein and its transcripts were increased after LPS stimulation. With the up-regulation of expression, PP4 is recruited into the receptor complex and interact with TRAF6, suggesting that PP4 is in the feedback loop of LPS/TLR4 pathway.

Protein phosphatases that catalyze the dephosphorylation of serine and threonine residues have been classified into four subtypes: PP1, PP2A, PP2B and PP2C. PP4 belongs to the

PP2A family which comprises of a subset of PP2A-like phosphatase that includes PP2A, PP4, PP5 and PP6. PP2A has been verified to inhibit the activation of NF-κB and PP6 is involved in negatively regulating the activity of TAK1 [24,25]. Recently, a large scale mapping of human protein–protein interactions has shown that regulatory A subunit and the catalytic subunit of PP2A can bind to TRAF6 [26], suggesting that PP2A may be involved in TRAF6-associated signaling transduction. In our experiments, we failed to detect any interaction between TAK1 and PP4 or IKKβ and PP4, moreover, TAK1- and IKKβ-induced NF-κB were not suppressed by co-expressed PP4, which lead us to suppose that PP4 could not inactivate TAK1 and IKKβ as PP6 or PP2A does. Previous studies on PP4 have defined that PP4 is a positive regulator that activates NF-κB and JNK [14,16]. As to the different observations obtained by us and others, we attribute them to the controversy roles of PP4 in distinct pathways and cell types. Whether PP4 acts as a phosphatase in this pathway and which protein serves as the substrate for PP4 need to be further elucidated. However, our findings may provide some new insights into PP4 activities in the innate immune system. Till now, no PP4-deficient mice were obtained because the ablation of PP4 leads to the embryonic lethality. It reveals the importance of PP4 in mammalian development and survival, meanwhile makes it more difficult to fully understand what PP4 exactly does in different cells.

The rapid response of TLRs to pathogen is essential for maintaining a healthy microenvironment, but the stepped-up inflammatory response is harmful to health in an uncontrolled state. Recently, a panel of negative regulators has been identified and a picture of how the immune balance in the mammalian host is achieved in TLR-mediated signal transduction is beginning to emerge. Interestingly, many of the negative regulators act to block the activation of IRAK-1 and TRAF6, which are considered as two pivotal adaptors that provide platforms for different signals to converge. It is of great importance to figure out how the proteins cooperate with each other and through which mechanism they are controlled. The precise negative regulations of TLR pathways utilizing by different cell types remain unknown, therefore any progress made in future will help us understand the immune regulation in human body better.

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